J. Clin. Chem. Clin. Biochem.
Vol. 25, 1987, pp. 363–367

Immunonephelometric Quantification of Free Haemoglobin

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(Received September 8, 1986/February 23, 1987)

Summary: A fully mechanized immunonephelometric method for rapid and specific determination of free haemoglobin in serum, plasma, or aqueous haemolysates is described. It utilizes commercially available rabbit antiserum against human haemoglobin and the Behring Nephelometer Analyser. A single determination is performed within 6 minutes and requires 250 µl total sample volume. The measuring range is about 9–2300 mg/l haemoglobin. Precision is characterized by intraassay coefficients of variation of 3.4% and 1.6%, and interassay coefficients of variation of 8.2% and 5.6% for haemoglobin concentrations of 29 mg/l and 119 mg/l, respectively. Accuracy of the method is shown by close correlation to haemoglobin determination by cyanohaemiglobin spectrometry (r = 0.9837). Interference may occur for visibly lipaemic samples. A preliminary comparison of different specimens drawn in parallel shows lowest haemoglobin concentrations in citrate plasma, followed by heparin plasma and by serum, while EDTA-plasma is unsuitable for this analysis.

Introduction

Immunochemical techniques are increasingly utilized for specific detection and quantification of the haemoglobin component of occult blood in stool (1–5); in addition, they have been used for detection of haemoglobinopathies (6–8) and for determination of glycated haemoglobin (HbA_{1c}) in blood (9) but have so far found little attention for determination of haemoglobin in plasma (10, 11). This analysis is still performed spectrometrically (12–16) or colorimetrically (17–21). The latter methods, which are based on the pseudoperoxidase activity of haemoglobin, are also widely used for detection of erythrocytes, or haemoglobin, in stool and urine (22, 23). They suffer from many types of interference (17, 22); e. g., by ascorbate (20, 23) and even by albumin (18). The specificity of several proposed methods has apparently not been assessed. The spectrometric haemoglobin determination may be subject to interference by bilirubin (12) and lipaemia (16), and has so far not been mechanized; it may however gain new acceptance with the advent of derivative spectrometry (15) or multi-wavelength diode-array spectrometers (24).

The previously described immunonephelometric procedures for determinations of haemoglobin in biological fluids are of low sensitivity (10) or low precision (11) and do not appear to have found application in routine diagnosis.

Faced with the clinical need of continuous haemolysis monitoring of intensive care patients with extracorporal circulation, we have attempted to develop a specific immunological method for quantification of free haemoglobin in serum and plasma. We present here a mechanized, simple, fast, and sensitive assay procedure, which requires only commercially available reagents and equipment for this straightforward parameter in haemolysis diagnosis and monitoring.

Materials and Methods

The antiserum used is rabbit antiserum raised against human haemoglobin A, not immuno-absorbed against haemoglobin F (lot Nos. 5243 and 7925, Behringwerke, Marburg, FRG). Monospecificity was checked by immunodiffusion according to *Ouchterlony* (25). Absence of cross-reactivity with myoglobin was established by Dr. *H. Vermeer*, Behringwerke (personal communication).

The assays were performed on the Behring Nephelometer Analyser (Behringwerke, Marburg, FRG), using the following parameter setting:

sample dilution:	1:5
sample volume:	50 µl
antiserum volume:	40 µl
supplement reagent volume:	15 µl
reaction buffer volume:	2 × 80 µl
reaction time:	6 min (fixed time kinetics with blank correction)

Haemoglobin in whole blood was determined by mechanized cyanohaemiglobin spectrometry on a TOA Sysmex CC-800 analyser (Colora, Lorch, FRG). Haemolysates as standards or accuracy controls were prepared by dilution of whole blood with pure water and centrifugation to remove the stroma. Haptoglobin was determined by immunonephelometry using the Behring Nephelometer and the provided test scheme. Human globin was from Sigma (Munich, FRG), human haptoglobin and Lipoclean® were from Behringwerke (Marburg, FRG). For precision control, the following control sera were used: N/T Protein Control Serum, Kontrollogen LU (both from Behringwerke, Marburg, FRG), Monitrol I (Merz + Dade, Munich, FRG), and Validate-A (Gödecke, Freiburg, FRG). The activity of the lactate dehydrogenase isoenzyme 1/2 (LDH 1/2, α-hydroxybutyrate dehydrogenase) was determined with a commercially available reagent kit (Monotest, Boehringer Mannheim, FRG) on a Cobas Bio analyser (Roche, Grenzach-Wyhlen, FRG).

Patient specimens were obtained by routine puncture of the antecubital vein. For preparation of serum, the blood was collected in plain tubes and was allowed to clot for about 1 h at room temperature, then centrifuged for 10 min at 3000 *g*. Serum was separated from the clot and stored at 4 °C until analysis (no longer than 24 h). Plasma specimen were obtained by collecting blood in tubes containing either solid K-EDTA, solid Na-heparinate, or 1 volume of Na-citrate solution per 9 volumes of blood. The tubes were centrifuged within 1 h, the plasma removed and stored at 4 °C until analysis.

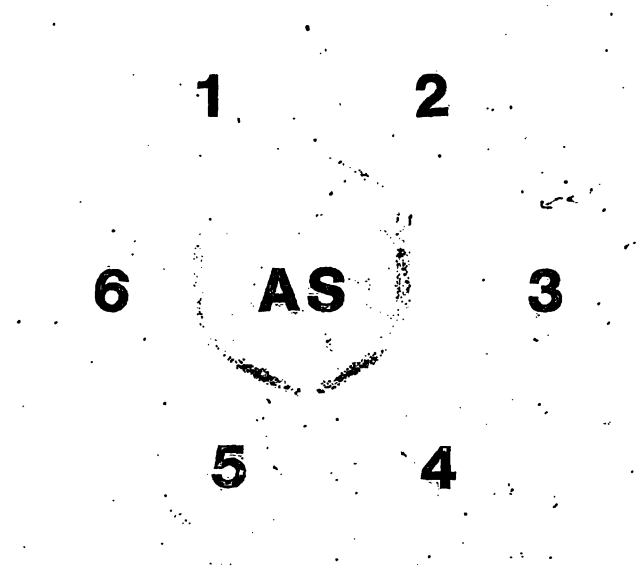

Fig. 1. *Ouchterlony* gel diffusion assay of LN plasma standard (well 1), LN plasma standard spiked with haemolysate to 260 mg/l haemoglobin (well 2), aqueous haemolysate with 2600 mg/l and 260 mg/l haemoglobin, respectively (wells 3 and 4), and patient sera (wells 5 and 6) against anti-human-haemoglobin antiserum (AS).

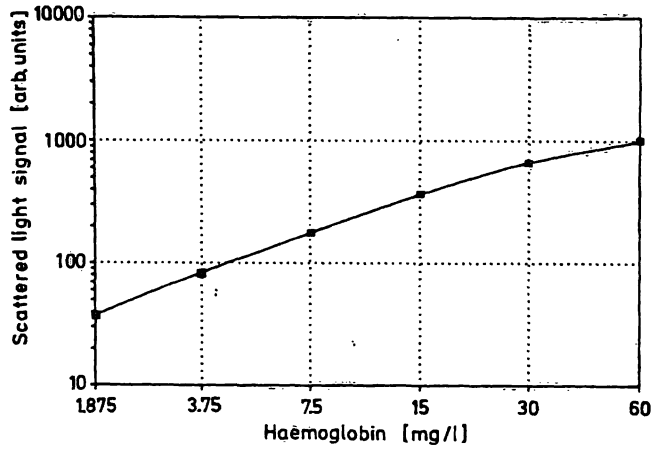

Fig. 2. Calibration curve of the haemoglobin determination with the nephelometer analyser. The haemoglobin concentrations of the standard dilutions (geometric dilution series from 1:10 to 1:320) (abscissa) and the intensity of the scattered light signal given in arbitrary units (ordinate) are shown.

Results

The commercially available antiserum is monospecific as shown by immunodiffusion against haemolysed whole blood, patient sera, and control sera (fig. 1). It is also precipitating, as shown by the calibration curve of the Nephelometer Analyser, which reports the scattered light intensity in dependence on antigen concentration (fig. 2). Thus it appears suited for quantitative immunonephelometric analysis. The calibration curve ranges from 9 mg/l to 300 mg/l free haemoglobin for samples (sample dilution 1:5). The *Heidelberger* curve for the haemoglobin-antihaemoglobin reaction shows the maximum scattered light signal at about 1500 mg/l haemoglobin and a signal equivalent to the highest calibration standard at about 2300 mg/l. Only samples with haemoglobin concentrations exceeding the latter figure are assigned falsely low results due to antigen excess; however, such rare cases are easily recognized visually by their intense red colour and should be analysed in manually selected higher sample dilutions (1:20, or 1:100). Samples with haemoglobin concentrations between the above mentioned limits are automatically re-analysed at the higher dilutions by the nephelometric analyser, thus yielding correct results. In conclusion, the total measuring range of the immunonephelometric haemoglobin-determination is about 9 mg/l to 2300 mg/l, greatly exceeding the clinically important range. It should be noted, however, that this measuring range is only achieved with antisera not immuno-absorbed against haemoglobin F, because otherwise the antibody titre is markedly lower.

The precision of the method was assessed by determining the within-series imprecision as well as the between-series imprecision for single determinations using pool sera of different haemoglobin content. The results are compiled in table 1. Similar precision data are obtained from the different dilutions of aqueous haemolysate standard used for calibration of the method (data not shown). The good precision at all concentration levels allows all analyses to be performed as single determinations. For routine purposes, commercially available control sera may be used for precision control, as these contain considerable amounts of immunoreactive haemoglobin.

Tab. 1. Precision of the immunonephelometric haemoglobin determination.

	Haemoglobin concentration (mg/l)	Imprecision (coefficient of variation, %)	Number of samples
Within series	29	3.4	20
	58	1.5	40
	119	1.6	20
Between series	27	8.2	26
	59	7.4	26
	119	5.6	26

For evaluation of accuracy, the immunonephelometric method was compared with the chemical determination of haemoglobin by cyanohaemiglobin-spectrometry. After analysis of the haemoglobin-content by the latter method, the samples were diluted 1000-fold with water. The haemolysed samples were centrifuged to remove the stroma, and the clear supernatants were subjected to immunonephelometric haemoglobin determination. The results (fig. 3) show close agreement of the methods. Serially diluted haemolysates and patient sera yielded linear results over the entire range from 600 to 9 mg/l haemoglobin. Mixing of haemolysates of different haemoglobin content, or of patient sera of different degrees of haemolysis demonstrated complete recovery. Slight differences were observed, however, when haemolysate was added to serum, or when serum was diluted with saline or water. These differences appear to be caused by protein matrix effects on the immunoreaction.

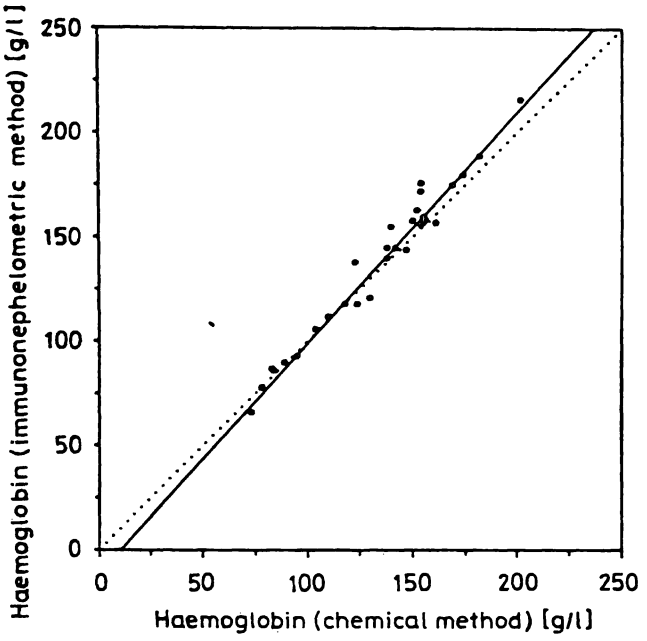

Fig. 3. Method comparison for haemoglobin determination in patient samples by cyanohaemiglobin spectrometry (abscissa) and by immunonephelometry (ordinate). The latter data are corrected for the appropriate dilution factor (1:1000). The linear regression function is $y = 1.0968\ x - 8.82$; coefficient of correlation $r = 0.9837$.

Lipaemic specimens, selected by their visible turbidity, were analysed before and after delipidization with Lipoclean®. On average, the apparent haemoglobin content of the turbid samples amounted to 160% of the concentration in their cleared counterparts, but severely lipaemic sera (triacylglycerols >10 mmol/l) occasionally yielded even falsely low results. Lipaemic samples with increased haemoglobin content mostly gave correct results, especially when analysed in 1:20 dilution. These findings are not specific for the haemoglobin assay as they also hold true for other Behring Nephelometer assays with 1:5 sample dilution and low analyte concentration. Even greater deviations occur when the lipaemia is not of endogenous origin, but results from lipid infusions in the course of parenteral nutrition (unpublished observations). The absence of interference in immunonephelometric haemoglobin determination from complexation of the antigen with haptoglobin has already been shown by *Engler* et al. (10). We have confirmed their finding by spiking aqueous haemolysates with pure human haptoglobin to physiological concentrations (1.5 to 2.0 g/l). The resulting immunoreactive haemoglobin concentrations amounted 90–110% of the starting concentrations.

The immunonephelometric determination of free haemoglobin requires a total sample volume of 250 µl. A single determination lasts 6 min, while large series are analysed at 225 samples per hour. The reagent costs are approximately DM 2.50 per test. No particular skill of the analyst is required.

In a preliminary attempt to define influence factors on free haemoglobin, we compared its concentration in different kinds of specimens, obtained in parallel from different populations of apparently healthy persons, or from patients (tab. 2). The lowest concentra-

Tab. 2. Comparison of free haemoglobin concentration and lactate dehydrogenase 1/2 activity in different kinds of specimen, and among different populations. For healthy persons, ambulatory patients, and blood donors venipuncture was performed by skilled staff, while for stationary patients the samples were drawn mostly by personnel in training.

Population	n	Free haemoglobin (mg/l)				Lactate dehydrogenase 1/2 (U/l)		
		Citrate plasma	Heparin plasma	EDTA-plasma	Serum	Citrate plasma	EDTA-plasma	Serum
Healthy persons	12	–	42 ± 12	624 ± 468	67 ± 24	–	–	–
Unselected stationary patients	12	16 ± 6	–	424 ± 205	154 ± 83	75 ± 13	143 ± 36	112 ± 17
Ambulatory patients	24	–	–	632 ± 417	69 ± 26	–	–	–
Healthy persons	92	–	–	–	67 ± 41	–	–	–
Blood donors, aliquot of sample	38	–	–	–	41 ± 26	–	–	–

tion of free haemoglobin and the least variation is seen in citrate plasma, followed by heparin plasma. Serum samples show considerable variation even among healthy persons and furthermore depend heavily on the conditions and skill of specimen collection. EDTA-plasma is totally unsuitable for determination of free haemoglobin, owing to severe in vitro haemolysis especially during centrifugation. Haemolysis is less severe, when the erythrocytes are allowed to settle by gravity before centrifugation. The low haemoglobin content in citrate plasma is not an analytical artifact, as mixtures of citrate plasma and haemolytic sera show complete recovery; their low degree of haemolysis is also obvious from visual inspection and from their low activity of lactate dehydrogenase 1/2, an established parameter of erythrocyte damage (tab. 2). Overall, the concentrations of haemoglobin and the activity of lactate dehydrogenase 1/2 are significantly correlated ($r = 0.852$, $p < 0.001$).

Discussion

We present here the evaluation of a fast, simple (owing to full mechanization), and precise method for determination of free haemoglobin, i. e., immunonephelometry. The nephelometric analyser can be integrated into the emergency laboratory and, in addition, allows simultaneous determination of two further haemolysis parameters, haptoglobin and haemopexin, from the same sample. We have proved the applicability of immunonephelometry to aqueous samples as well as to serum and plasma, and thus it should also be suited for haemoglobin determination in other biological fluids like urine, liquor cerebrospinalis, or gastric fluid, provided the concentration is sufficiently high. Our method is inherently of higher specificity than the currently used procedures. Consequently, haemolysed patient samples show very good correlation to the long-established reference method for haemoglobin determination in whole blood, i. e., cyanohaemiglobin spectrometry (fig. 3). There is no interference from reducing substances (drugs, ascorbate) which inhibit the pseudoperoxidase reaction of colorimetric haemoglobin determinations (16, 19), or from bilirubin which may disturb spectrometric assays (12). Only visibly lipaemic samples are unsuitable for the immunonephelometric haemoglobin determination, since the sample dilution is only 1:5; they should be delipidized before analysis.

Even the presence of haptoglobin which forms a complex with haemoglobin has only minor influence on the immunoreactivity of the molecule. We have not checked whether this is a property of the antiserum or whether the complex is dissociated in the reaction mixture by the detergent-containing supplement reagent. As a consequence, the method is equally well suited for samples from patients with normal haptoglobin levels, and for patients with chronic or severe haemolysis and haptoglobin-deficient serum. In most other methods for free haemoglobin quantification the effect of haptoglobin has apparently not been assessed. As expected, the antihaemoglobin-antiserum also reacts with free globin (data not shown). This should not limit the specificity of our proposed method, since we are not aware of the occurrence of free globin in serum.

When applying the immunological haemoglobin determination to serum or plasma it should be realized that the presence of total plasma proteins in the reaction mixture slightly attenuates the nephelometric signal as compared with aqueous haemolysed samples. This effect may of course be circumvented by spiking standard sera or pool sera with haemoglobin and using them as calibration standards. This approach, however, makes preparation of standards and accuracy controls more cumbersome and more expensive. In addition, it requires a second standardization method for free haemoglobin determination instead of cyanohaemiglobinometry of whole blood. We have therefore continued to use aqueous haemolysed standards and controls.

Table 2 demonstrates that much more work is required on influence factors on the concentration of free haemoglobin. The specimen of choice appears to be citrate plasma, even when not meticulously prepared as recommended for heparin plasma (16), and even though its results have to be corrected for a dilution factor. Besides heparin plasma, serum might also be suitable, but depends heavily on the conditions and skill of venipuncture. EDTA-plasma cannot be used, probably because EDTA labilizes the erythrocyte plasma membrane. Studies on reference ranges and the clinical value of the determination of free haemoglobin are likely to yield meaningful results only after these issues have been clearly resolved. Our method should greatly facilitate such future work.

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